Developmental and Comparative Immunology (2008) **32**, 1497–1509

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Shrimp single WAP domain (SWD)-containing protein exhibits proteinase inhibitory and antimicrobial activities

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Received 13 May 2008; received in revised form 5 June 2008; accepted 7 June 2008
Available online 3 July 2008

KEYWORDS
Shrimp;
Penaeus monodon;
Whey acidic protein domain;
Crustin;
Proteinase inhibitor;
Antimicrobial peptide

Summary
Single WAP domain (SWD)-containing proteins are small proteins with a C-terminal region containing a single whey acidic protein (WAP) domain. In the present study, the cDNAs representing three isoforms of SWD proteins (SWD*Pm*1, SWD*Pm*2 and SWD*Pm*3) were identified from hemocytes of the black tiger shrimp, *Penaeus monodon*. The deduced peptides revealed that they contain a putative signal peptide of 24 amino acids and encode for a mature peptide of 69, 68 and 56 amino acids, respectively, which contain typical characters similar to those of the shrimp SWD proteins (type III crustin) with a Pro-Arg region and a WAP domain towards the C-terminus. Tissue distribution analysis by RT-PCR showed that all three SWD*Pm* transcripts were primarily found in hemocytes. Transcript expression of SWD*Pm*1 was down-regulated upon injection with *Staphylococcus aureus* whilst there was no change of SWD*Pm*2 and SWD*Pm*3 expression. In contrast, white spot syndrome virus (WSSV) injection resulted in a biphasic response with up-regulation of SWD*Pm*1 and SWD*Pm*2 transcripts at 6 h followed by significant down-regulation by 24 h after infection. Genomic organization of the SWD*Pm*2 gene revealed the presence of three exons interrupted by two introns. To characterize the biological functions of the SWD protein, the mature SWD*Pm*2 protein encoding cDNA was cloned and expressed in *Escherichia coli*. Purified recombinant (r)SWD*Pm*2 exhibits antibacterial activity against several Gram-positive, but not Gram-negative, bacteria and is a competitive inhibitor of subtilisin A with an inhibition constant (K_i) of 1.98 nM. Thus, rSWD*Pm*2 may contribute to the inhibitory regulation of subtilisin A from bacterial infection and *P. monodon* SWD

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0145-305X/$ - see front matter
doi:10.1016/j.dci.2008.06.005

protein likely function as immune effectors in defense against invasion of shrimp pathogens.

Introduction

Innate immunity in multicellular organisms is the first line of defense against invading microbes such as bacteria, fungi and viruses. Like other invertebrates, shrimp do not have an acquired immunity; instead they have an efficient innate immune system, which includes melanization by the prophenoloxidase-activating system, a clotting process, phagocytosis, encapsulation of foreign material, cell agglutination and a diverse array of general and specific antimicrobial peptides (AMPs) [1].

AMPs and proteinase inhibitors are important components of the host innate immune system and play crucial roles in the host defense against microbial invasion. AMPs, including penaeidins [2], antilipopolysaccharide factors [3] and crustins [4,5] are found in shrimp hemocytes. Serine proteinase inhibitors, which are widely distributed in animals, plants and microorganisms, play a critical role in the regulation of many biological processes. Unsurprisingly then, many pathogens are known to produce extracellular proteinases which are reported to serve an active role in the development of various diseases. Several reports have suggested that a major function of proteinase inhibitors is to combat the proteinase of pathogens [6], and it is no surprise that at least some proteinase inhibitors have been found in crustaceans, such as members of the Kazal [7–9], serpin [10], pacifastin [11] and alpha 2-macroglobulin [12–16] families. These proteinase inhibitors and AMPs both serve defensive roles and exert the effect on pathogens.

Proteins containing a whey acidic protein (WAP) domain, initially characterized as a milk protein, have been found in several species of vertebrates and invertebrates [17,18]. The WAP domain comprises of approximately 50 amino acids including eight cysteine residues which form a four-disulfide core (4-DSC) [17]. The WAP domain is not however exclusive to WAP proteins but is found in numerous other proteins, where it may be present as multiple domains. WAP domain proteins are, however, typically small secretory proteins, which exhibit a variety of functions including proteinase inhibitory and antimicrobial activities [19,20].

Currently, a family of single WAP domain (SWD)-containing proteins in crustaceans has been described as crustins [18]. Type I and II crustins have been characterized in several species of crustaceans and have been shown to be abundant and to exhibit antimicrobial activity mainly against Gram-positive bacteria. Type III crustin classification is based on the protein domain structure which contains a signal peptide and a Pro-Arg region at the N-terminus and a WAP domain towards the C-terminus. Currently, the presence of Type III crustin has only been reported in shrimp and no biological function has yet been reported. The SWD-containing proteins from *Litopenaeus vannamei* (SWD*Lv*) and *Penaeus monodon* (SWD*Pm*) have been identified and current studies have mainly focused on cDNA sequences, gene expression levels and promoter analysis [21–23]. Here, we report the cDNAs encoding three isoforms of the SWD-containing protein from the black tiger shrimp, *P. monodon*, and for the first time, the biological functions were ascribed to this shrimp, or at least the *P. monodon*, SWD protein (type III crustin).

Material and methods

Animals and sample preparation

Three-month-old subadult black tiger shrimp, *P. monodon*, of about 20 g weight, were obtained from a local farm in Thailand. The challenge experiment was performed by injection into the last abdominal segment of each shrimp *either* shrimp salt solution (SSS: 450 mM NaCl, 10 mM KCl, 10 mM HEPES, pH 7.3) as a control, or a suspension of formalin-inactivated *Staphylococcus aureus* (10^8 CFU) in the same volume of SSS. Alternatively, shrimp were challenged *with* a viable white spot syndrome virus (WSSV) virion suspension (7.6×10^5 viral copies of WSSV) in lobster hemolymph medium (LHM), prepared as previously described [24], or with the same volume of LHM alone (control). Hemocytes were collected from the shrimp ventral sinus at 0, 6, 24 and 48 h after *S. aureus*, WSSV and control (SSS or LHM only) injections into an anticoagulant solution of 10% (w/v) trisodium citrate dihydrate pH 4.6. Collected hemolymph was immediately centrifuged at 800*g* for 10 min at 4 °C to separate hemocytes from the plasma and the hemocyte pellet was then immediately resuspended in TRI REAGENT® (Molecular Research Center, USA) for further processing.

RNA isolation and first-strand cDNA synthesis

Total RNA was isolated from dissected tissues, including hemocytes prepared as above, using TRI REAGENT® and treated with DNase I (Promega, USA) following the manufacturer's protocol. Total RNA concentration and integrity was assessed by UV spectrophotometry and agarose gel electrophoresis. First-strand cDNAs were synthesized from 2 μg of DNA-free total RNA sample and 0.5 μg of oligo $(dT)_{18}$ primers using the ImProm-II™ Reverse Transcriptase System kit (Promega, USA) according to the manufacturer's protocol.

Tissue distribution analysis

RT-PCR was carried out to investigate the transcript expression profile of SWD*Pm*1, SWD*Pm*2 and SWD*Pm*3 transcripts in different tissues of *P. monodon* including hemocytes, hepatopancreas, lymphoid organ, gill, intestine and heart. A pair of each SWD*Pm*-specific primers (SWD*Pm*1-F and SWD*Pm*1-R, SWD*Pm*2-F and SWD*Pm*2-R, and SWD*Pm*3-F and SWD*Pm*3-R) was designed (Table 1). The elongation